

Ultra High Sensitivity Analysis of Testosterone

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¹Michal Star-Weinstock, ¹Brian L. Williamson, ¹Subhakar Dey, ¹Babu Purkayastha, ²James E. Hill, ²Jeanette R. Hill
¹AB SCIEX, 500 Old Connecticut Path, Framingham MA, 01701
²Spot On Sciences, 13705 Shadowglade, Manor, TX 78653

ABSTRACT

- **Analysis of Testosterone (Te) from both Free Te samples (FTe) and Dried Blood Spots (DBS) utilizing derivatization with a novel aminoxy reagent is demonstrated**
- **The LLOQ after derivatization is <1 pg/mL for FTe samples and ~40 pg/mL for DBS**
- **Linearity ($R^2 > 0.998$) is maintained over a broad dynamic range with %CV <15 at LLOQ**
- **QAO-derivatization and sample preparation workflows are quick, simple, reproducible, robust and yield high recoveries**

INTRODUCTION

Testosterone (Te) analysis by LC/MS/MS is becoming the analytical method of choice over immunoassays due to its specificity and accuracy. Neutral steroid hormones undergo poor ionization efficiency in MS/MS, resulting in insufficient sensitivity to analyze samples which possess trace concentrations. The method presented herein utilizes a tag moiety (quaternary aminoxy reagent) which binds to the keto functionality of Te or any other keto-steroid and significantly enhances its MS/MS sensitivity. The derivatization technique enables quantitation of Free Te (FTe) from samples such as equilibrium dialysates, ultra filtrates and saliva. Moreover, the derivatisation technique enables the use of very small amounts of sample such as in Dried Blood Spots (DBS).

REAGENTS AND METHODS

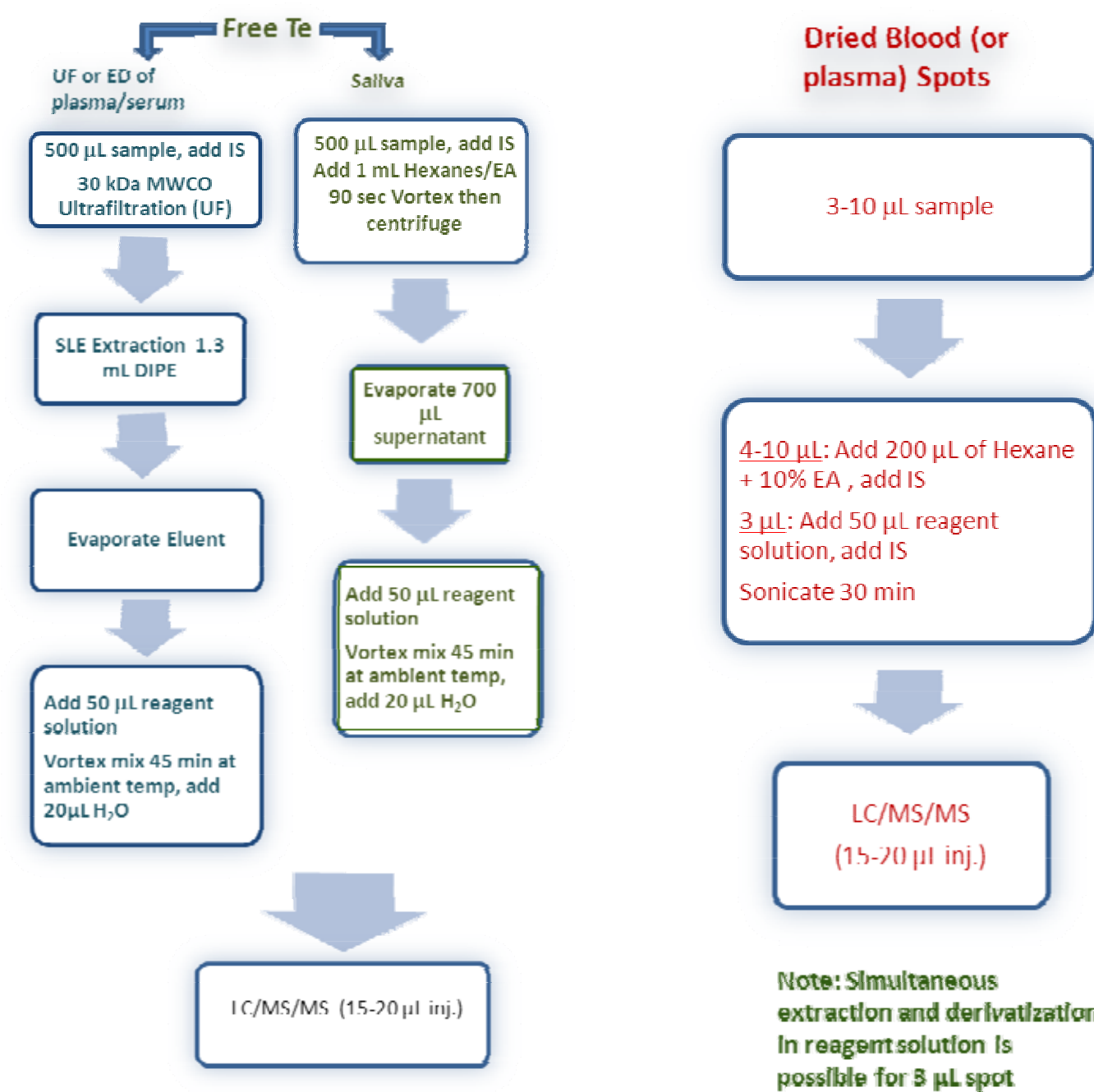


Figure 1. Schematic description of the extraction and derivatization workflow for Free Te samples from serum or Saliva and Dried Blood Spots

The QAO reagent (10 mg/mL), is dissolved in MeOH:5% acetic acid. d₃ Te is used as calibrator (Cerrilant, 99.8% pure). ¹³C Te used as Internal Standard (IS), Isosciences, >98% pure. Matrix: Female whole serum (Golden West Bio PS 1040). LC Gradient Conditions: Acetonitrile / water / formic acid (0.1%) mobile phase using a C18 column (Cadenza CL). Step Gradient 10-30%B in the first 0.5 min, then 30-55%B within 3 min. Shimadzu AD-30 series and QTRAP® 5500 LC/MS/MS system.

Results

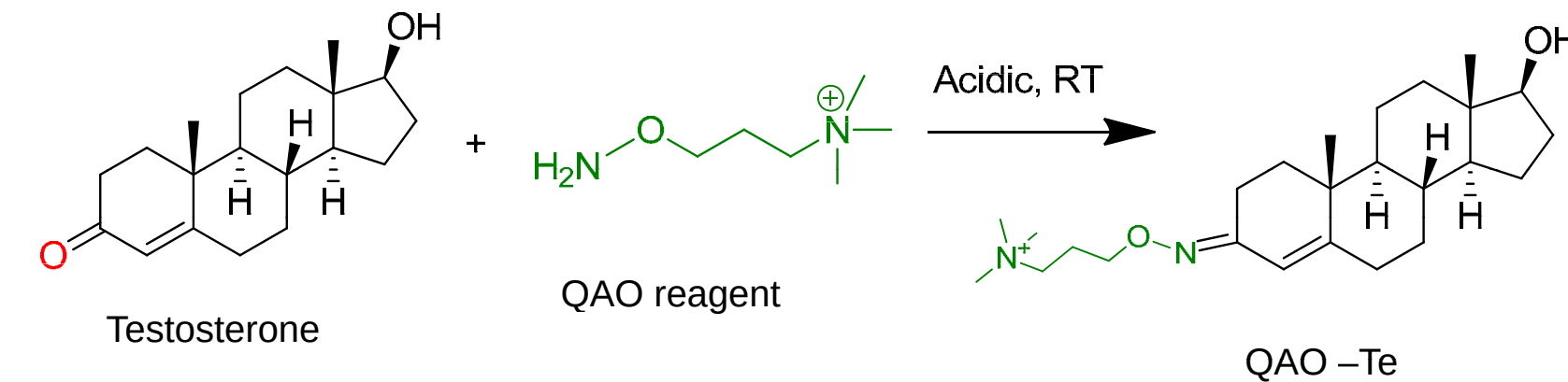


Figure 2. Schematic illustration of QAO derivatization of Te.
The reaction is complete within 45 min at RT

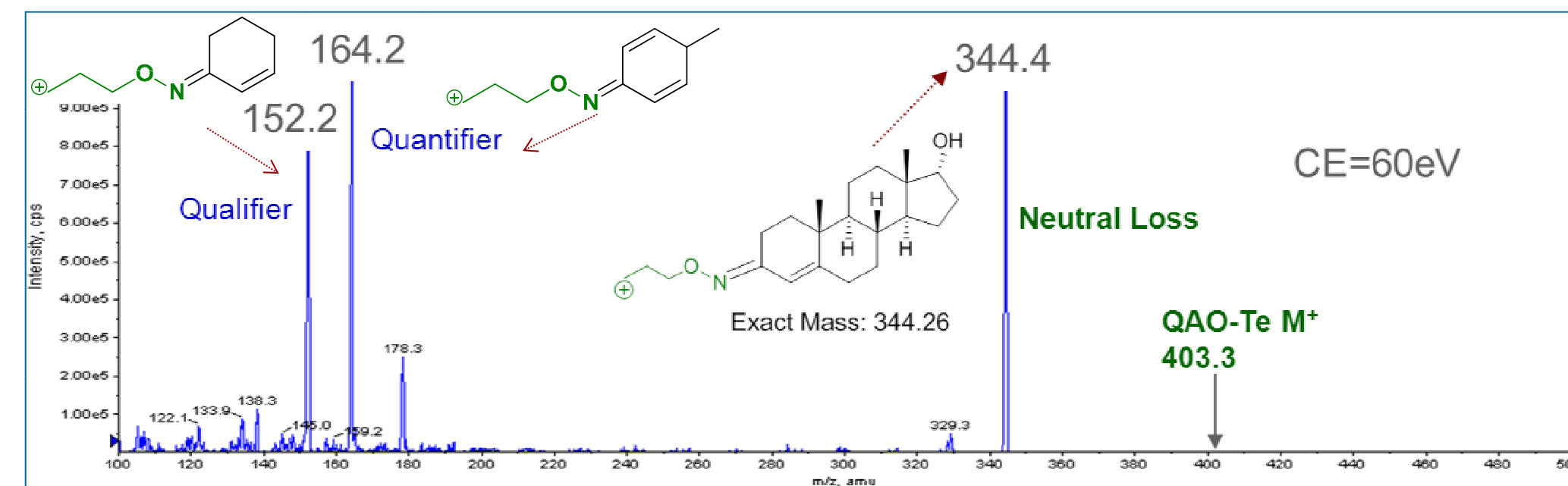


Figure 3. MS/MS fragmentation of QAO Te.

Structure specific fragments are selected to enhance specificity and reduce background noise. The MRM Transitions for QAO- Te are 403.3->164.2 and 403.3->152.2 at CE=60eV

Figure 4 illustrates the sensitivity enhancements after derivatisation of Te (1pg on column). Underivatized, derivatized with Hydroxyl amine and derivatized with QAO reagent were all compared. The S/N ratio of the QAO derivative is superior due to careful selection of specific fragment ions and higher mass of the derivative that drives it farther away from the background noise.

Notes:

1. Offsetting of retention times were used to position the peaks for visual demonstration.

2. The Hydroxyl amine (HA) Derivatization of Te was repeated from Kushnir MM. et al. Clinical Chemistry 56:7,1138–1147 (2010)

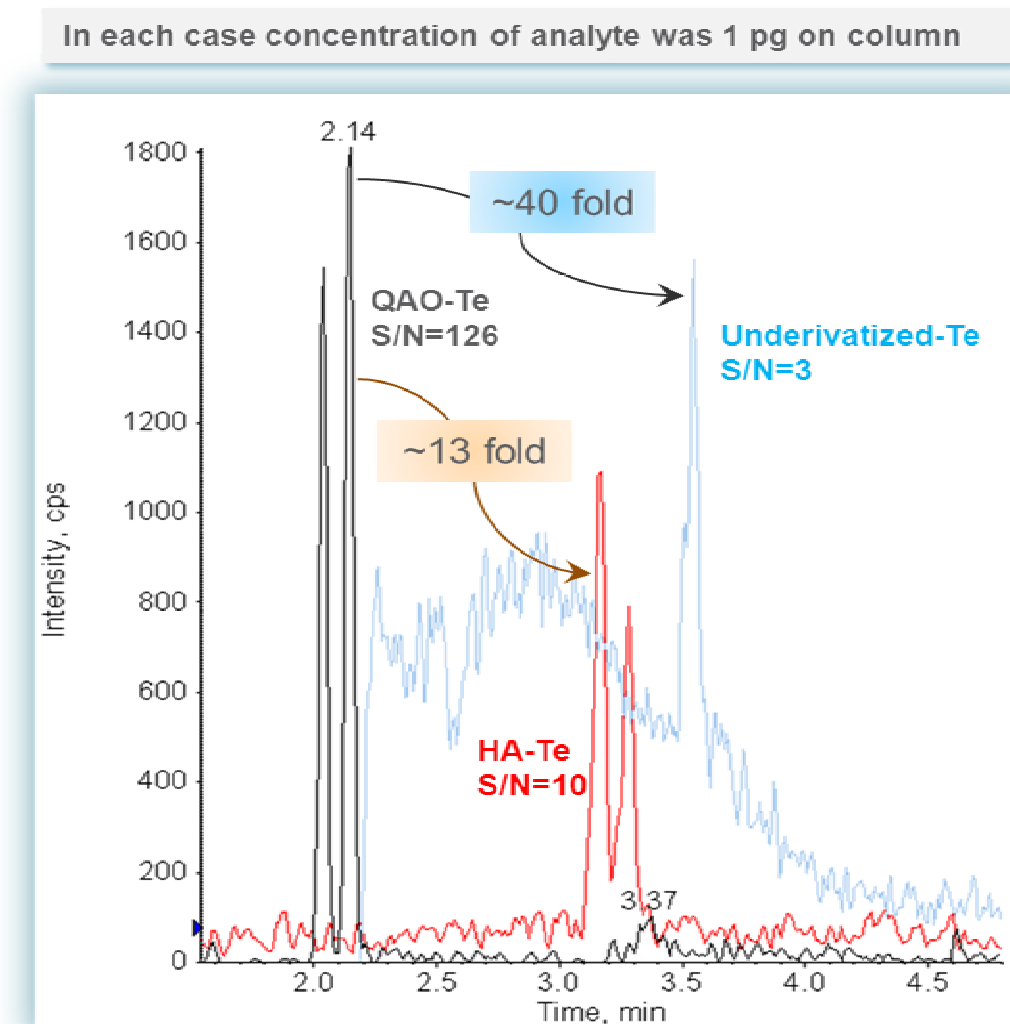


Figure 4. Sensitivity enhancement of Testosterone upon derivatization using QTRAP® 5500 LC/MS/MS.

Total Te from Dried Blood Spots (DBS)

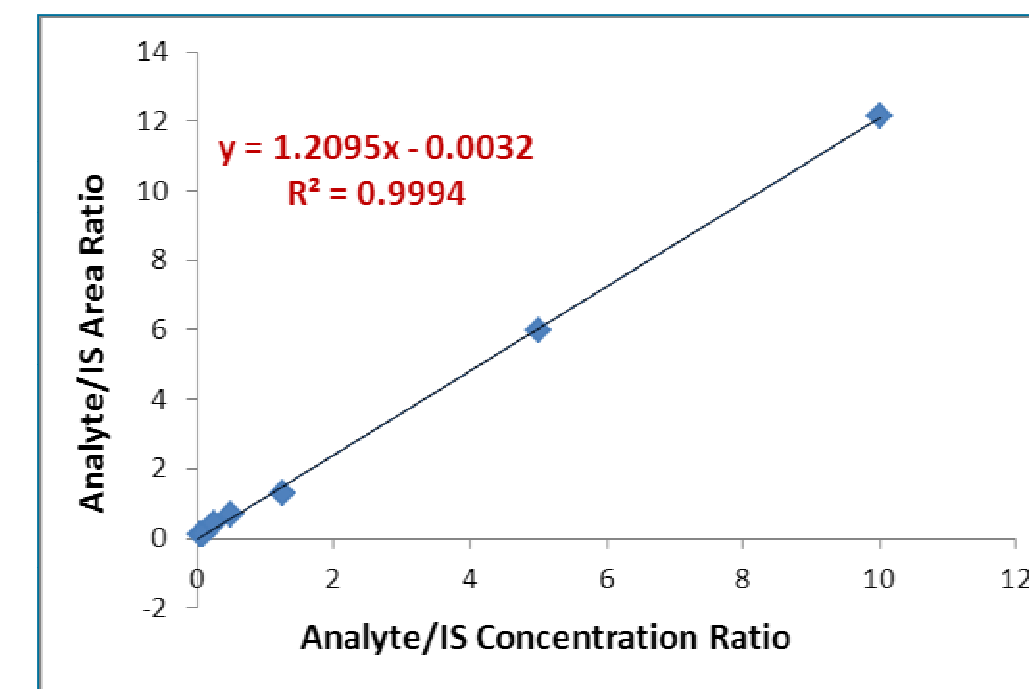


Figure 5. DBS Concentration curve of d₃ Te 50-10000 pg/mL spiked into 10 µL whole blood

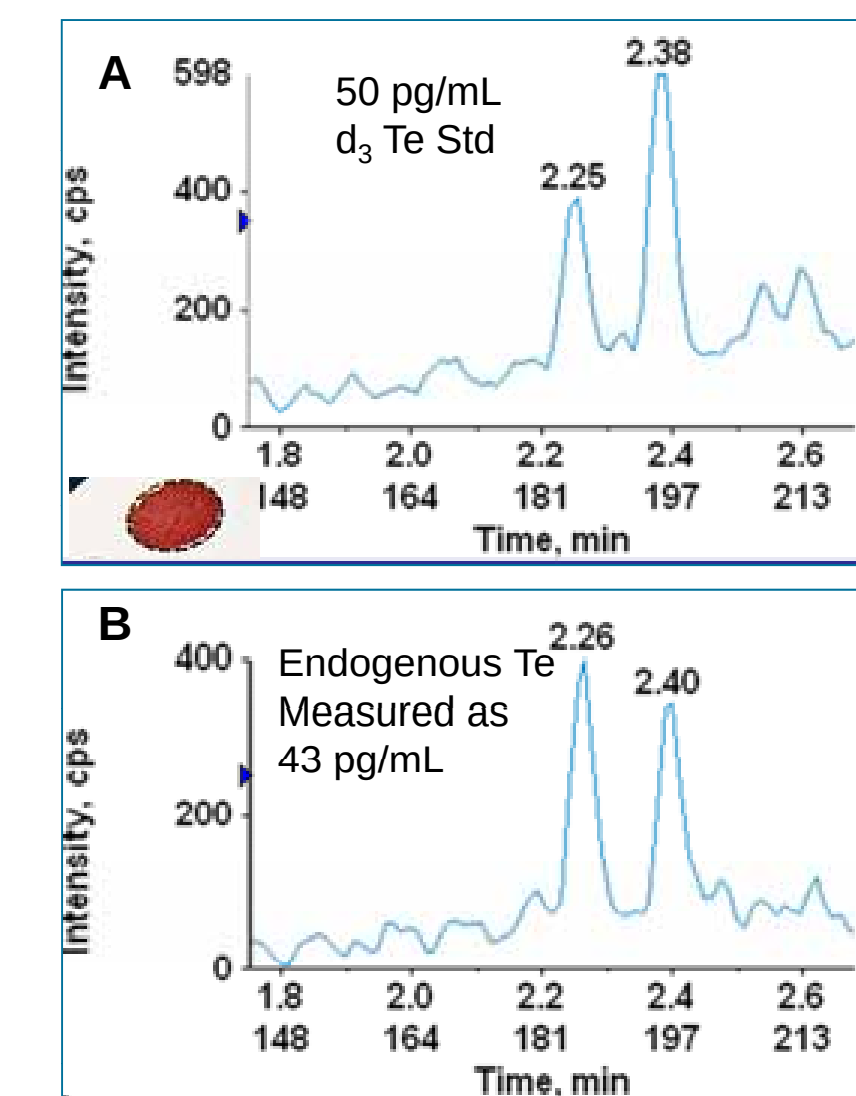


Figure 6: 10 µL DBS from female blood
A. d₃ Te Standard spiked as 50 pg/mL (0.5 pg in 10 µL blood, on a conventional filter paper)

B. Endogenous Te in the same DBS sample which was calculated as 43 pg/mL

Reproducibility at the LLOQ level of ~43 pg/mL: %CV= 10.7 (n=22)

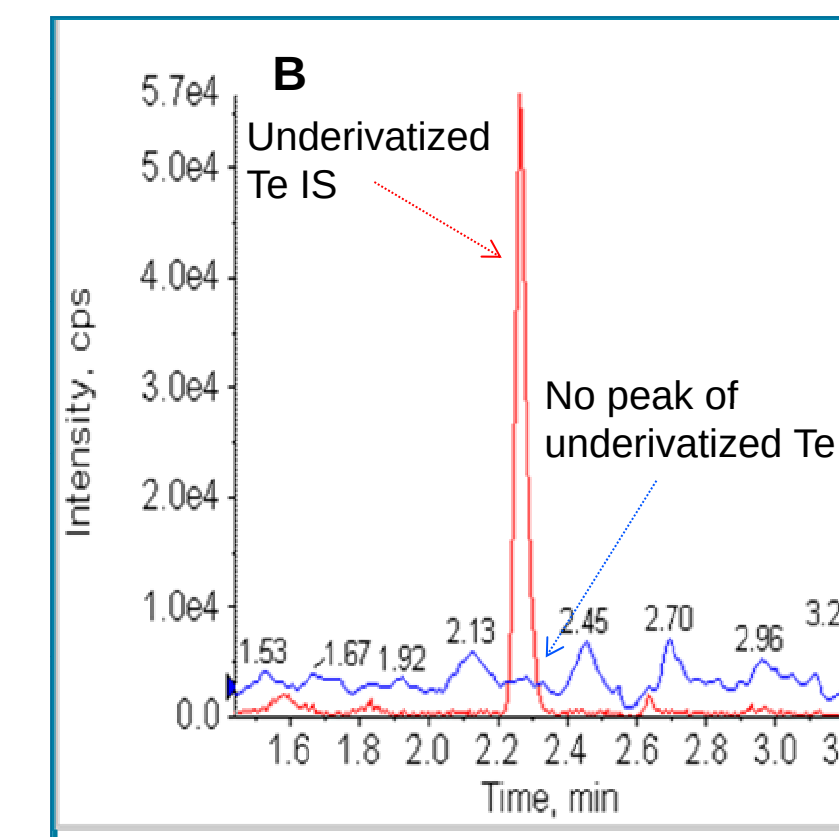
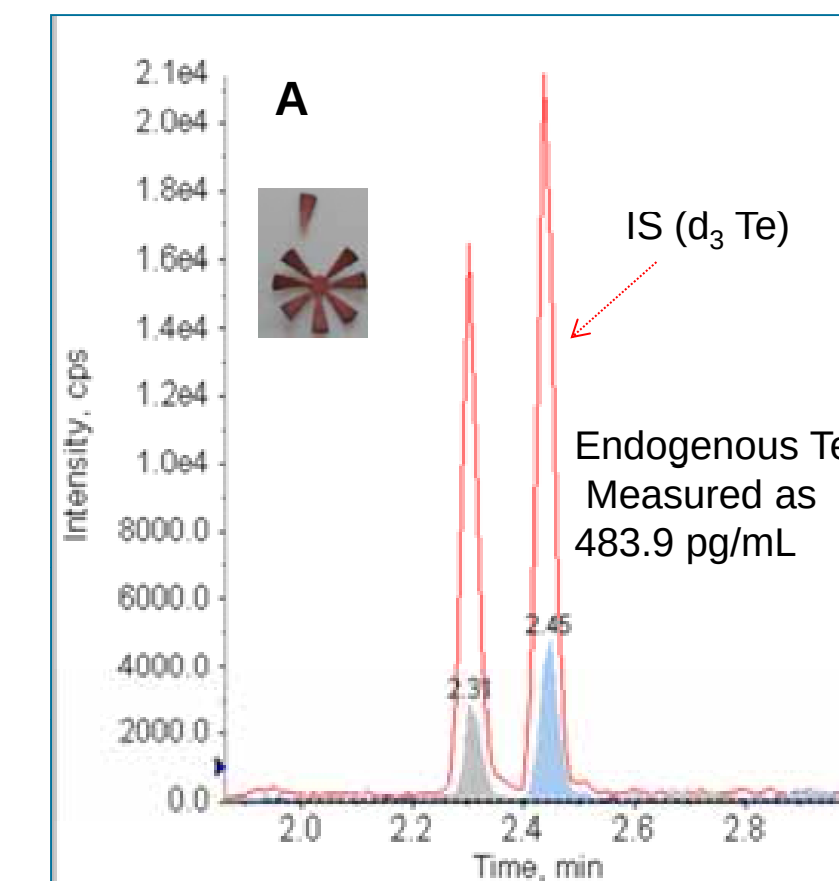


Figure 7: Chromatogram of a DBS sample from a 28 year old female: (A) following derivatisation, (B) underivatised

The sample is extracted from a HemaSpot fan shaped filter paper, ~8 µL whole blood in each blade is extracted with 200 µL Hexane/10% ethyl acetate as describe in Figure 3.

Free Te From Serum or Saliva

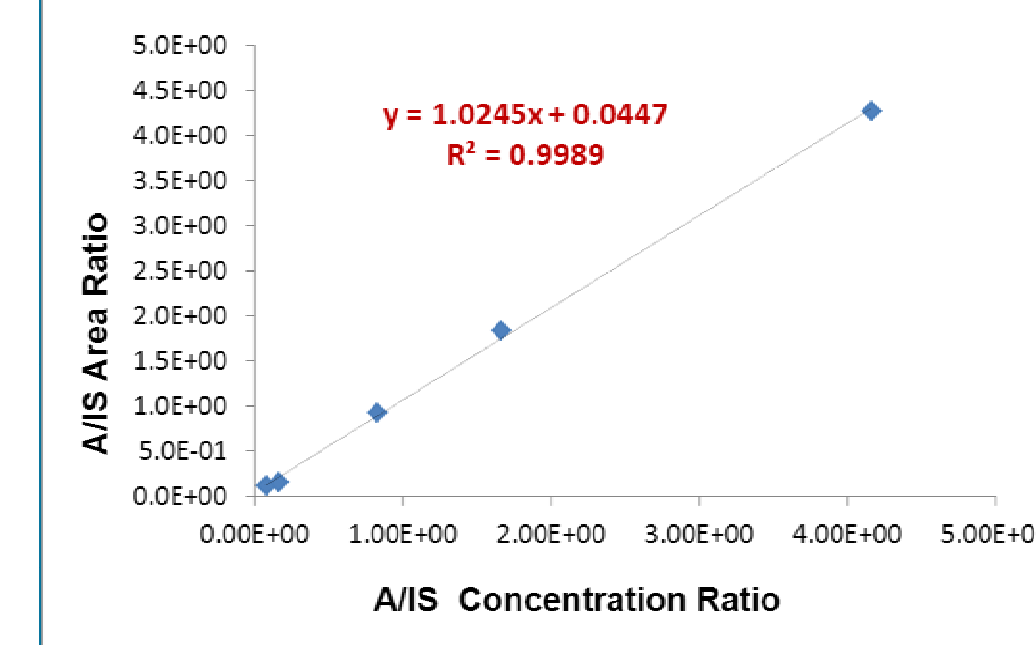


Figure 8. Concentration curve 1-50 pg/mL, calibrator spiked into ultra filtrate MWCO 30 kDa of female serum pool (500 uL). The calibrator was d₃ Te and the IS ¹³C Te.

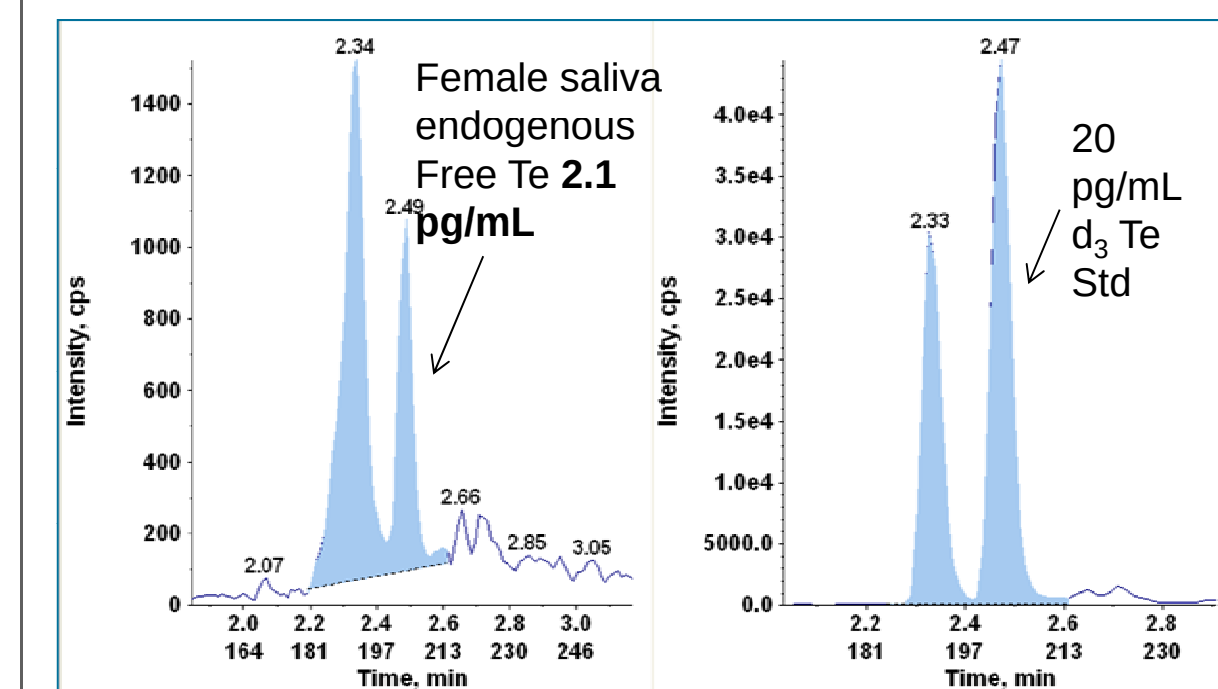


Figure 9. Ultra filtrate (30kDa MWCO) of Female serum pool spiked with ¹³C Te IS 10 pg/mL. The endogenous FTe was measured as 0.9 pg/mL. Since the Total Te concentration was 82.7 pg/mL, the % Free Te ≈1.14

Reproducibility: At this concentration level of ~1pg/mL the % CV = 14.7 (n=15)

Figure 10. Free Te analysis from female saliva.
1 mL saliva was extracted by the method described in Fig. 3.

Conclusions

- Derivatization of Te with a novel aminoxy reagent (QAO) leads to improved LC-MS/MS properties
- This derivatisation leads to a significant increase in ESI/MS/MS Te sensitivity which enables analysis of Te in Dried Blood Spots (<10µL) and Free Te samples.
- The LLOQ levels achieved with the QAO reagent (<1pg/mL for FTe samples and <50 pg/mL for DBS) are a significant improvement over previously reported values
- QAO reagent is universal and reacts well with other compounds containing a keto or aldehyde functionality.

TRADEMARKS/LICENSING

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